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13. ABSTRACT ***(Maximum 200 Words)***
PTHrP was discovered as the tumor product responsible for the syndrome of humoral hypercalcemia of malignancy. Experiments in the first two years of this project have shown that PTHrP has an important role during embryonic mammary development. In the third year of this project we have gained a deeper understanding of PTHrP's functions in the embryonic development of the mammary gland. PTHrP is expressed within epithelial cells, during the early stages of mammary bud formation. The PTHrP receptor is expressed throughout the sub-epidermal mesenchyme. In those mesenchymal cells closest to the mammary epithelial bud, PTHrP induces a change in cell fate allowing those cells to become functional mammary mesenchymal cells. If PTHrP is absent, these cells differentiate into dermal cells that are incapable of supporting further epithelial development, and the expected morphogenesis of the mammary gland fails to occur. In addition, we have demonstrated that transient overexpression of PTHrP during embryonic development of the mammary gland has profound consequences for the branching pattern produced by the mammary epithelial ducts during adolescent development. This again underscores the fact that, during the embryonic formation of the mammary gland, PTHrP exerts powerful effects on mammary morphogenesis. Finally, we have begun to study the effects of PTHrP overexpression on DMBA-induced mammary tumorigenesis in mice.

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FOREWORD

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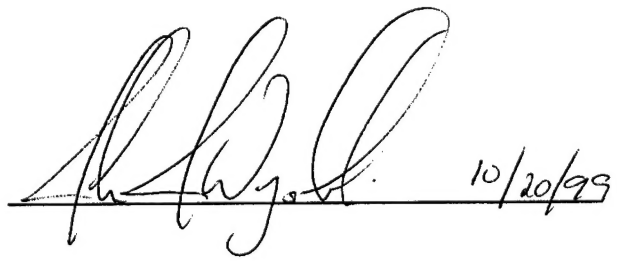

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Introduction

Parathyroid hormone-related protein (PTHrP) was originally discovered as a tumor product responsible for causing the clinical syndrome of humoral hypercalcemia of malignancy (HHM) (1). In this syndrome, PTHrP is released into the circulation by malignant cells, resulting in a typical constellation of biochemical abnormalities resembling hyperparathyroidism. The similarity of HHM and hyperparathyroidism is now understood on a molecular basis. The parathyroid hormone (PTH) and PTHrP genes arose by duplication from a common ancestral gene and continue to share a high degree of homology in their amino-terminal ends, a feature that allows them to signal through the use of a common receptor (termed the PTH/PTHrP type I receptor) (1,2). In the setting of malignancy, PTHrP, which normally acts as a local autocrine or paracrine factor, is secreted into the circulation by tumor cells and interacts with PTH/PTHrP receptors in bone and kidney, mimicking the actions of PTH (1).

As noted in the preceding paragraph, PTHrP normally acts as a local autocrine and/or paracrine factor. It is expressed in a great number of tissues, where it appears to play a role in the regulation of cellular proliferation and differentiation during development (3). One of these sights is the mammary gland. PTHrP mRNA has been shown to be expressed in the embryonic mammary epithelium, as well as by mammary epithelial cells during pregnancy and lactation (3,4). In addition, PTHrP has been shown to be secreted by mammary myoepithelial cells in culture (5,6). The PTH/PTHrP receptor is found on mammary stromal cells and on mammary myoepithelial cells (4-6). Therefore, the mammary gland contains the elements of both autocrine and paracrine signaling loops for PTHrP. That these loops are important to the physiology of mammary development is evident by the results of the overexpression of PTHrP in mammary myoepithelial cells (7). As reviewed in the original proposal, we used the human keratin-14 (K14) promoter to target PTHrP overexpression to myoepithelial cells. This resulted in a severe impairment of branching morphogenesis and mammary ductal proliferation during sexual maturation and pregnancy (7). These results led us to hypothesize that PTHrP acts as a local growth inhibitor, contributing to the regulation of ductal proliferation and morphogenesis during mammary development.

In recent years, accumulating evidence has suggested that PTHrP is important to the establishment of bone metastases in breast cancer. This work has come primarily from the laboratory of Dr. Theresa Guise in San Antonio. Using a mouse model in which bone metastases are scored following the introduction of tumor cells into the arterial circulation, she has demonstrated that the level of PTHrP production by human breast cancer cell lines influences their ability to form osteolytic bone metastases (8). That is, the more PTHrP the tumor cells make, the greater the number and size of the bone metastases. It appears that the ability of the cells to make PTHrP in response to TGF-β found in the bone microenvironment may be particularly important to their ability to form bone metastases (9). There is currently conflicting data regarding the ability of PTHrP expression in a primary breast tumor to predict subsequent bone metastases in humans (10,11). It may be that the upregulation of PTHrP within the bone microenvironment is

more important to this process than the level of PTHrP production in the primary tumor itself.

The intent of the current grant is to test the hypothesis that PTHrP serves as a locally-produced mammary growth inhibitor by examining the effects of PTHrP on branching morphogenesis and on mammary epithelial cell proliferation and transformation. In order to test this hypothesis we proposed a series of four technical objectives that encompassed a mixture of experiments *in vitro* and in transgenic animals. As summarized in previous reports, over the last three years, we have made great strides in understanding PTHrP's function during embryonic mammary development. We know that PTHrP is necessary for normal mammary development. It appears to be an important participant in the normal epithelial-mesenchymal conversation that regulates the formation of the neonatal mammary gland. In PTHrP or PTHrP receptor knockout mice, there is a failure of the normal pattern of sexual dimorphism during mammary development, a failure of the initiation of branching morphogenesis and the mammary epithelial cells die. We have learned that these phenotypes result from a failure of the mammary mesenchyme to differentiate properly, and that PTHrP serves as a critical epithelial signal that the mammary mesenchyme needs in order to become functionally competent in supporting mammary epithelial development. Having characterized these embryonic effects of PTHrP, over the past year, we have turned our attention back to its effects during the pubertal development of the gland as well as its effects on mammary tumor formation and metastasis. These findings are presented below, organized by technical objective.

BODY

Technical Objective 1. – Effects of the loss of PTHrP on mammary gland development

As described in last year's report, we had discovered that PTHrP signaling via the type I PTH/PTHrP receptor was necessary for the androgen-mediated destruction of the mammary bud. To review, an important aspect of embryonic mammary development is the sexual dimorphism that occurs after the formation of the mammary bud. In female embryos the mammary bud remains relatively quiescent from its formation on E12 until the initiation of branching morphogenesis on E16. In male embryos, however, the fetal testes begin to make androgens on E13, which leads to the destruction of the mammary buds. In response to androgens, the mammary mesenchyme condenses around the neck of the mammary bud and, by E14, severs its connection with the epidermis (12). In most strains of mice, the mammary epithelial cells in males subsequently degenerate by E15 - E16. This process has been studied in some detail and it is known to rely on a series of epithelial-mesenchymal interactions. First, androgen receptor expression is induced in the mammary mesenchyme between E12 and E14 under the direction of the mammary epithelial cells (13). The mesenchymal cells are the cells that subsequently respond to fetal androgens, and they, in turn, sever the epithelial stalk and destroy the mammary epithelial cells (14,15). Our experiments have shown that PTHrP is the signal that is sent from epithelium to mesenchyme and which results in the induction of androgen receptor

expression in the mammary mesenchyme. Furthermore, we believe that PTHrP induces a fundamental change in cell fate in the mammary mesenchyme, distinguishing these cells from the ventral dermal mesenchyme and allowing them to direct subsequent epithelial morphogenesis. Since PTHrP is expressed only within the developing epithelial bud during embryonic mammary development, while its receptor is expressed generally within the sub-epidermal mesenchyme, our hypothesis is that PTHrP acts as a dominant signal contributing to the differentiation of the specialized mammary mesenchyme in the immediate vicinity of the mammary epithelial bud (16).

We realized that we could test this hypothesis by examining androgen receptor and tenascin C expression within the dermis of our K14-PTHrP transgenic mice. As shown in Fig.1, Keratin 14 is expressed within the basal keratinocytes of the epidermis and thus, in transgenic embryos, PTHrP would now be expressed in keratinocytes as well as within the mammary epithelial bud. If our hypothesis that PTHrP was a dominant signal leading to the differentiation of mammary mesenchyme as opposed to dermal mesenchyme was true, then one would expect that in the K14 transgenics, the dermis would express markers of the mammary mesenchyme. As shown in Fig. 1, this indeed was the case. In sections of K14-PTHrP ventral skin, the androgen receptor was inappropriately expressed in the dermal mesenchymal cells nearest to the basal keratinocytes. Similarly, there was inappropriate tenascin C staining in these same cells. Interestingly, this was only true for the ventral surface of the K14-PTHrP mice, as similar sections of dorsal skin showed neither androgen receptor nor tenascin C staining (Fig. 1, D and G). This was not simply due to a lack of transgene or PTH/PTHrP receptor expression within the dorsal skin, as shown in Fig. 2. Furthermore, these findings agree with old unpublished data from Kratochwill and Robinson (shared by T. Robinson), showing that while mammary epithelial buds were able to induce androgen receptor expression in ventral dermal mesenchyme they could not do so in mesenchyme derived from dorsal skin.

In summary, data acquired over the last 2 years has allowed us to formulate the following model of PTHrP's actions in embryonic mammary development (outlined in Fig. 3). PTHrP expression is initiated specifically within the mammary epithelial cells of the mammary bud, as the bud is forming. The PTH/PTHrP receptor is expressed within all the mesenchymal cells underneath the fetal epidermis. PTHrP acts as an epithelial signal that contributes to the differentiation of the mesenchyme around the mammary epithelial bud and is necessary for that mesenchyme to acquire the ability to direct further morphogenesis of the mammary gland. Although all the sub-epidermal mesenchyme expresses the PTHrP receptor, only the ventral mesenchyme seems to be responsive to these mammary mesenchyme –inducing effects of PTHrP. If the ventral mesenchyme sees PTHrP, it expresses mammary mesenchyme markers and if it does not see PTHrP, then it differentiates into dermis.

These experiments were not part of the original proposal, as they followed from data that was unknown at the time of its submission. These findings served as the preliminary data for a NIH RO1 proposal, which was funded and will be starting this fall. This proposal will examine, in great detail, the mechanisms by which PTHrP causes these

cells to differentiate, and no further experiments in this regard will be performed under the auspices of this current grant. The results discussed in the preceding paragraphs were published in Development this summer.

Technical Objective 2. - The effects of PTHrP on the branching morphogenesis of mammary epithelial cells

The central hypothesis underlying this technical objective is that PTHrP regulates epithelial-stromal interactions that support the proper morphogenesis of the mammary epithelial duct system during the pubertal development of the mammary gland as well as during embryogenesis. As summarized in the original proposal and in last year's report, there are two lines of evidence that would support this concept. First, transgenic mice overexpressing PTHrP have an impairment in branching morphogenesis of the epithelial ducts that manifests itself at the time of puberty. Second, during the pubertal growth of the mammary gland, PTHrP is expressed in the epithelial cells of the end-buds and the PTH/PTHrP receptor is expressed in the stromal cells surrounding the end buds. End buds are specialized structures at the tips of the growing ducts where active cell proliferation, differentiation and forward growth of the duct system actually occurs. We had originally planned to embark upon a series of experiments *in vitro* designed to examine the effects of PTHrP on the ability of mammary epithelial cells to form branching tubes in three dimensional culture systems. However, given our emerging knowledge of the powerful effects of PTHrP on the differentiation of the mammary stroma, we first decided to ask if the changes in our transgenic mice represented an alteration in stromal differentiation during development or rather a physiologic effect of PTHrP during the pubertal growth phase. Stated another way, we asked if our phenotype was the result of prior embryonic overexpression of PTHrP or a result of PTHrP overexpression during puberty itself.

We approached this question by recreating our K14-PTHrP transgenic mouse, but this time employing a binary tetracycline-regulated system. As outlined in Figure 4, we used a "tet-off" system so that the addition of tetracycline into the water supply of the mice shuts off transgene expression. We created two lines of transgenic mice; K14-tTA mice expressed the tetracycline transactivator under the control of the K14promoter, and ptet-PTHrP mice expressed the PTHrP gene under the control of a minimum promoter with several tetracycline-responsive elements. Next, we bred these mice together to generate double transgenics, which overexpressed PTHrP at sights of K14 expression as before, but now with transgene expression dependent on the presence or absence of tetracycline in the animals' water. In other words, the transgene now had an on-off switch. The ability to turn transgene expression on or off enabled us to examine the consequences of PTHrP overexpression at different points in the development of the mammary gland.

Before examining the effects of varying the timing of PTHrP overexpression, we first bred our K14-tTa mice to a pTet-βGal responder mouse in order to track the expression of the K14-tTA transgene and to examine the ability of tetracycline to suppress transgene expression. In K14-tTA/pTet-βGal double transgenic mice, cells

expressing the tet-transactivator should stain blue with Xgal, but loose this property when tetracycline is present. As shown in Fig 5, as expected, the K14-tTA transgene is expressed in all mammary epithelial cells at birth and in myoepithelial cells in adults, but βGal expression is completely suppressed by the addition of tetracycline to the animals water.

Having validated our experimental system, we next examined the effects of PTHrP expression at different points in mammary gland development. Our first experiment was to examine the effects of PTHrP expression only before puberty vs. during puberty. Because the K14-PTHrP mammary phenotype becomes manifest during puberty, we had expected that concurrent exposure to PTHrP was necessary for this phenotype to emerge. Therefore, we were surprised to discover that expression of PTHrP up until the time of puberty, but not during puberty was sufficient to cause the K14-PTHrP phenotype, but overexpression of PTHrP during puberty, in the absence of prior PTHrP expression, was not associated with any abnormalities. Hence, it appears that exposure to PTHrP before the start of the adolescent growth spurt was able to cause abnormal growth even though PTHrP transgene expression was turned-off during the time in which the growth process was taking place. Next, we asked if embryonic expression was the key determinant of the K14-PTHrP transgenic phenotype. In these experiments we compared mammary epithelial growth in mice that always overexpressed PTHrP, never overexpressed PTHrP, overexpressed PTHrP only before birth, or overexpressed PTHrP only after birth. As expected, mice never overexpressing PTHrP had normal mammary development, while those always overexpressing PTHrP recapitulated the original K14-PTHrP phenotype (Fig. 6). As summarized in the original proposal, these mice demonstrated a severe delay in the penetration of the epithelial ducts through the mammary fat pad as well as a dramatic reduction of the branching complexity with many fewer secondary and tertiary branches. Interestingly, in the other two arms of this experiment, we saw a separation of these two aspects of the original phenotype. In mice overexpressing PTHrP only before birth, we noted a severe reduction in the branching complexity of the mammary duct system, but no significant delay in the growth of the ducts into the fat pad (Fig. 6). However, in mice overexpressing PTHrP only after birth, we observed the delay in the growth of the ducts into the fat pad, but no significant reduction in the branching complexity (Fig. 6). Therefore, these results suggest that the phenotype of the K14-PTHrP mice represents the combination of two separate effects of PTHrP on the pubertal growth of the mammary epithelial duct system. The first is a patterning defect that depends on embryonic overexpression of PTHrP and leads to a reduction in ductal sidebranching. We currently believe that this is the result of changes in the stroma induced by early exposure to excess amounts of PTHrP. The second is a slowing of the rate of ductal elongation and penetration into the fat pad and this aspect of the phenotype does depend on the concurrent overexpression of PTHrP during puberty. These results agree with our prior observations that placement of PTHrP pellets in advance of the growing duct system led to an inhibition of ductal elongation but not branching (7). They are also consistent with the results of our localization studies during puberty (detailed in last year's report) which detected high levels of PTHrP and PTH/PTHrP receptor mRNA in terminal end buds, the structures where the rate of ductal elongation is regulated (17). We feel that these studies provide further evidence

supporting the importance of PTHrP in determining the morphogenetic capacity of the mammary stroma during embryonic development. They also suggest that, in addition, PTHrP participates in a local regulatory loop at the terminal end buds which is involved in the regulation of epithelial proliferation and/or apoptosis.

These experiments were not part of the original proposal and thus are not a part of the original or revised Statement of Work. We are technically behind schedule with respect to our current Statement of Work. We put these experiments on hold pending the outcome of the experiments described above. Obviously, given these results we no longer plan to examine the morphological effects of PTHrP added to the media of co-cultures of stromal cells and EpH4K6 cells. Instead, we will concentrate on the effects of PTHrP on the proliferation of these cells as well as normal mammary epithelial cells, in co-culture with stromal cells as outlined under Technical Objective 3.

Technical Objective 3 – Examination of the effects of PTHrP on hormonally-induced proliferation in mammary epithelial cells.

Because of the new experiments described above, we have not started these experiments and are thus behind schedule. However, given our findings that PTHrP does appear to regulate epithelial duct elongation during puberty, experiments examining its effects on proliferation and/or apoptosis of mammary epithelial cells will be a high priority in the coming year.

Technical Objective 4 – The effects of PTHrP on mammary tumor formation.

As summarized in last year's report, and in the revised Statement of Work, we completed the experiments involving GR mice that were part of the original proposal. It did not appear that PTHrP production inhibited tumor formation in that model. In the time that has passed since the original proposal was written, it has been proposed that PTHrP may be important to the process of forming bone metastases from breast cancer. In order to test this hypothesis as well as to examine our original hypothesis that PTHrP would inhibit tumor formation in another model, we treated a cohort of our transgenic animals and controls with DMBA to examine chemical-induced transformation. We are currently in the middle of this experiment although similar to the GR-mouse model, it does not appear that PTHrP overexpression will protect these mice from forming mammary tumors.

We are on time with these experiments, having completed those originally proposed and having initiated those contained within the revised Statement of Work. We hope to complete the analysis of the DMBA-treated mice in the coming year.

KEY RESEARCH ACCOMPLISHMENTS FOR YEAR 3

1. Further elucidation of the effects of PTHrP on mesenchymal cell fate decisions during embryonic mammary development.
2. Creation and validation of tetracycline-regulated system for overexpression of genes in mammary epithelial cells
3. Use of above tetracycline-regulated system to separate the effects of PTHrP overexpression into developmental and concurrent effects.
4. Demonstration of the patterning defects in adolescent mammary gland development in K14-PTHrP transgenic mice to be the result of embryonic exposure to PTHrP.
5. Demonstration of the defects in ductal elongation in K14-PTHrP transgenic mice to be due to concurrent exposure to PTHrP during adolescence.

REPORTABLE OUTCOMES

Manuscripts:

1. Dunbar ME, Young P, Zhang JP, McCaughern-Carucci J, Lanske B, Orloff J, Karaplis A, Cunha G, Wysolmerski JJ. Stromal cells are critical targets in the regulation of mammary ductal morphogenesis by parathyroid hormone-related protein (PTHrP). *Developmental Biology,* 203: 75-89, 1998.

2. Dunbar ME and Wysolmerski JJ. Parathyroid hormone-related protein: a developmental regulatory molecule necessary for mammary gland development. *J Mammary Gland Biol Neoplasia,* 4:21-34,1999.

3. Dunbar ME, Dann PR, Robinson GW, Hennighausen L, Zhang JP, Wysolmerski JJ. Parathyroid hormone-related protein is necessary for sexual dimorphism during embryonic mammary development. *Development*, 126:3485-3493, 1999.

Abstracts/Presentations:

1. Dunbar M, Dann P, Robinson T, Wysolmerski J. PTHrP signaling is necessary for sexual dimorphism during fetal mammary development. Bone 23:5 (supplement), S 163, 1998. Oral presentation at combined ASBMR-IBMS joint meeting 12/98.

2. Dunbar M, Young P, Zhang JP, Lanske B, Orloff J, Karaplis A, Cunha G, Wysolmerski J. Stromal cells are critical targets in the regulation of mammary ductal morphogenesis by PTHrP. Bone 23:5 (supplement), S444, 1998. Poster presentation at combined ASBMR-IBMS joint meeting 12/98.

3. "PTHrP regulates fetal mammary development by modulating mesenchymal cell fate decisions", Henneker, New Hampshire, Gordon Research Conference on Mammary Gland Biology, 6/9/99.

4. "PTHrP regulates mammary ductal morphogenesis", San Diego, CA., Basic Symposium: Signaling Molecules in Breast Cancer, Endocrine Society Annual Scientific Meetings, 6/14/99.

5. Dunbar M, Dann P, Dreyer B, Broadus AE, Philbrick WM, Wysolmerski JJ. Transient early overexpression of PTHrP leads to subsequent defects in mammary development. Oral presentation , Endocrine Society Annual Scientific Meetings, 6/99.

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CONCLUSIONS

This year, as in the past several years, we have learned more about the effects of PTHrP on embryonic mammary development. We have extended our findings from last year on the relationship between PTHrP expression and androgen receptor and tenascin C expression during embryonic mammary development by showing that ectopic expression of PTHrP within the epidermis leads to conversion of the dermis into mammary mesenchyme. It appears that there is a zone of responsiveness consisting of the developing ventral epidermis, in which PTHrP is able to influence cell fate decisions within the sub-epidermal mesenchyme and lead to mammary mesenchyme differentiation as opposed to dermal mesenchyme differentiation. Since PTHrP is expressed at high levels only within the mammary epithelial bud, it, in effect, acts as a patterning molecule to insure that the epithelial bud becomes invested with functional mammary mesenchyme. This difference in cell fate has implications for the subsequent morphogenesis of the epithelium, because only the mammary mesenchyme can support the proper development of the epithelial structures. It is clear that the next step in furthering our understanding of PTHrP's effects on embryonic mammary development will come from a detailed examination of the mechanisms by which PTHrP alters the cell fate of the relatively undifferentiated mesenchyme. The data gleaned from our studies into the PTHrP knockout mice have formed the preliminary data for a NIH-funded proposal to do just this.

Studies this past year have also refined our understanding of the effects of PTHrP overexpression on the development of the mammary gland during puberty. It is clear from our studies in tetracycline-regulated transgenic mice that our original phenotype represented the combination of patterning defects due to PTHrP's effects on embryonic mammary development as well as effects of PTHrP on ductal elongation occurring during the pubertal growth phase. Based on localization studies from last year that showed that PTHrP and its receptor are highly expressed in terminal end-buds during puberty, we believe that PTHrP participates in a local regulatory loop between epithelium and stroma that regulates ductal elongation at the terminal end bud. Studies in progress are attempting to determine if this represents effects on cell proliferation, apoptosis or both. As a result of these data, in the coming year, we will not pursue studies examining the ability of PTHrP to affect branching morphogenesis in culture, but will rather concentrate on studies examining its ability to regulate cell proliferation.

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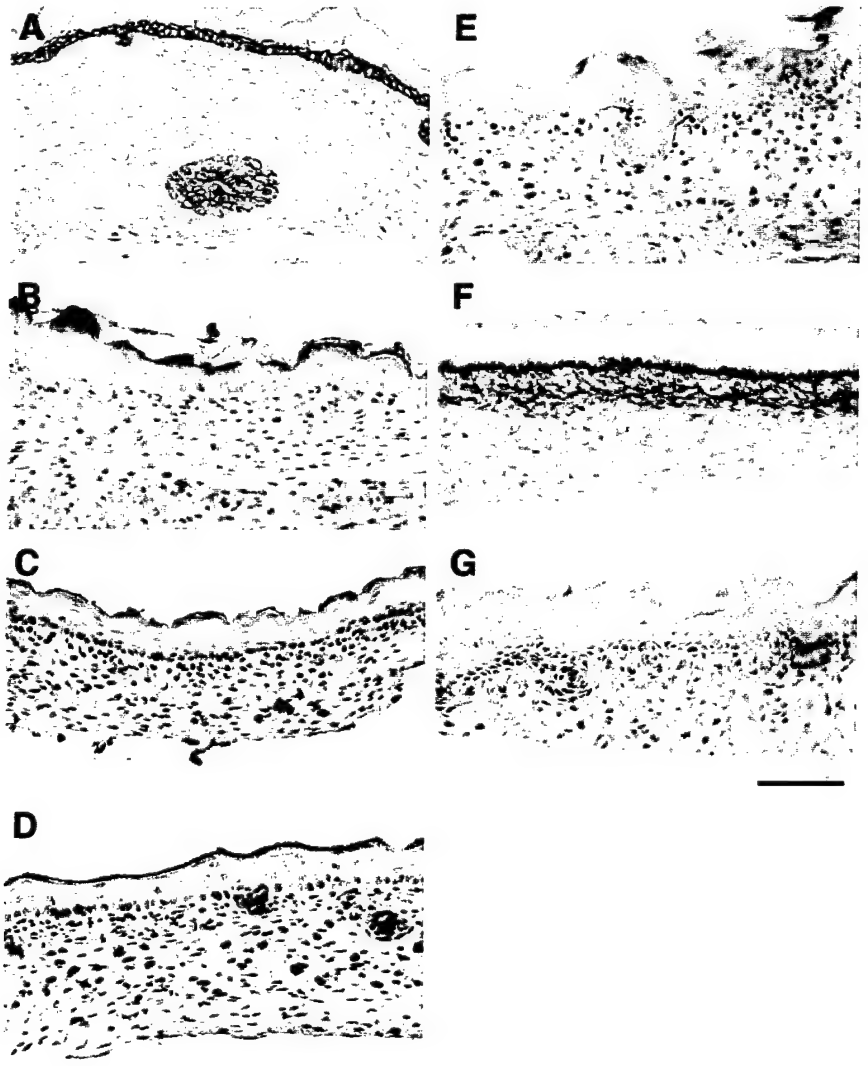
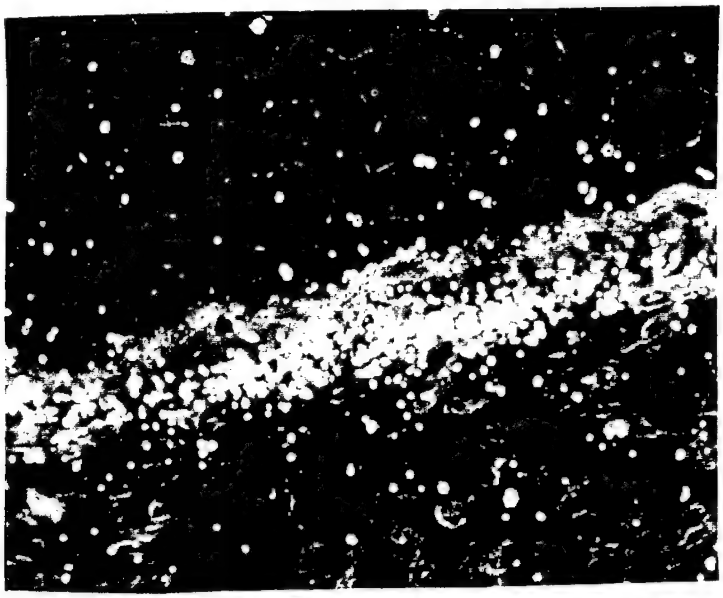


Figure 1. Ectopic overexpression of PTHrP in the epidermis induces dermal expression of androgen receptor and tenascin C. (A) Immunohistochemistry for keratin 14 in the fetal epidermis at E18. Note that K14 is expressed both within the fetal mammary epithelial cells (arrow) and within the basal keratinocytes of the epidermis. (B-D) Androgen receptor staining of ventral epidermis from a wild-type embryo at E18 (B), and of ventral (C) and dorsal (D) epidermis from a K14-PTHrP transgenic embryo, also at E18. There is no androgen staining in the wild-type dermis (B), but there is nuclear androgen receptor staining in the dermal cells close to the epidermal basement membrane in the ventral surface of the K14-PTHrP transgenic. However, this is not true for the dorsal aspect of the K14-PTHrP embryos as seen in (D). A similar pattern is seen for tenascin C (E-G). In the ventral surface of wild-type embryos at E18 (E), there is some tenascin expression along the basement membrane, especially in the vacinity of developing hair follicles. However, there is a dramatic upregulation of tenascin within the basement membrane and within the extracellular matrix of the upper dermis on the ventral surface of K14-PTHrP embryos at E18 (F), but not on the dorsal surface of K14-PTHrP transgenic embryos (G).

A



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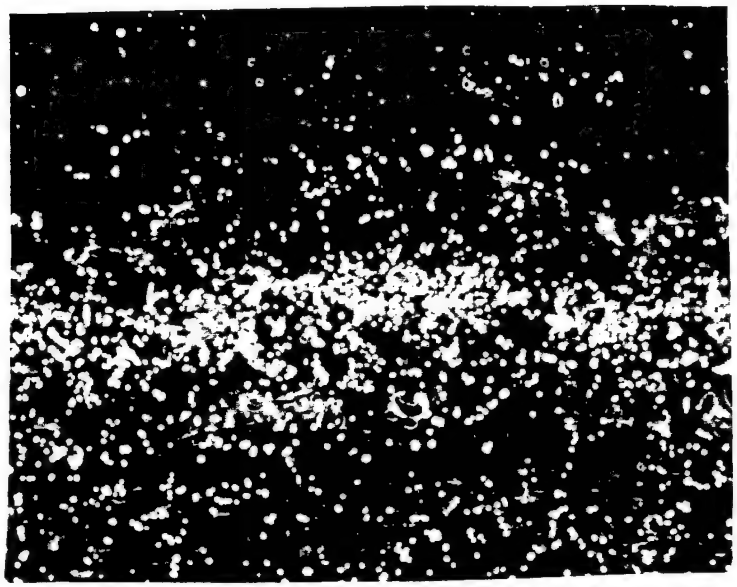


Figure 2. Detection of K14-PTHrP transgene and PTH/PTHrP mRNA expression in dorsal skin of mice by in situ hybridization. (A) Dark-field view of a section of dorsal skin taken from a K14 PTHrP transgenic mouse and hybridized with radio-labeled antisense probe for the transgene. As can be seen, despite the lack of induction of androgen receptor and tenascin C expression in the dorsal skin of these mice, transgene expression is present. (B) Dark-field view of a section of dorsal skin taken from a K14-PTHrP transgenic mouse and hybridized with a radio-labeled antisense probe for the PTH/PTHrP receptor gene. As can be seen, there is ample receptor expression in the dermis of the doral aspect of the skin of these mice. Therefore, the absence of ectopic androgen receptor and tenascin C expression in the dorsal skin of these transgenic mice cannot be explained simply by the lack of transgene or receptor expression.

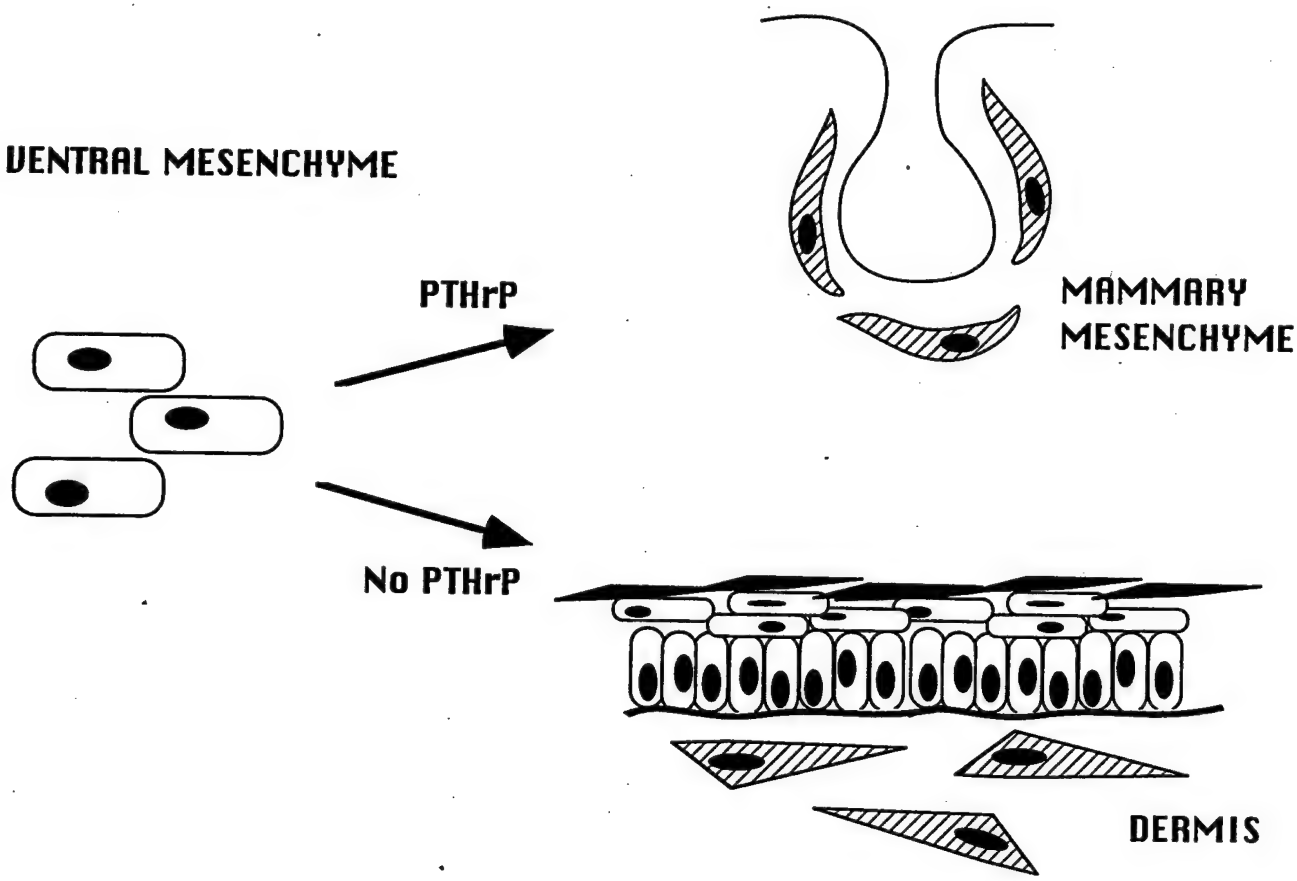
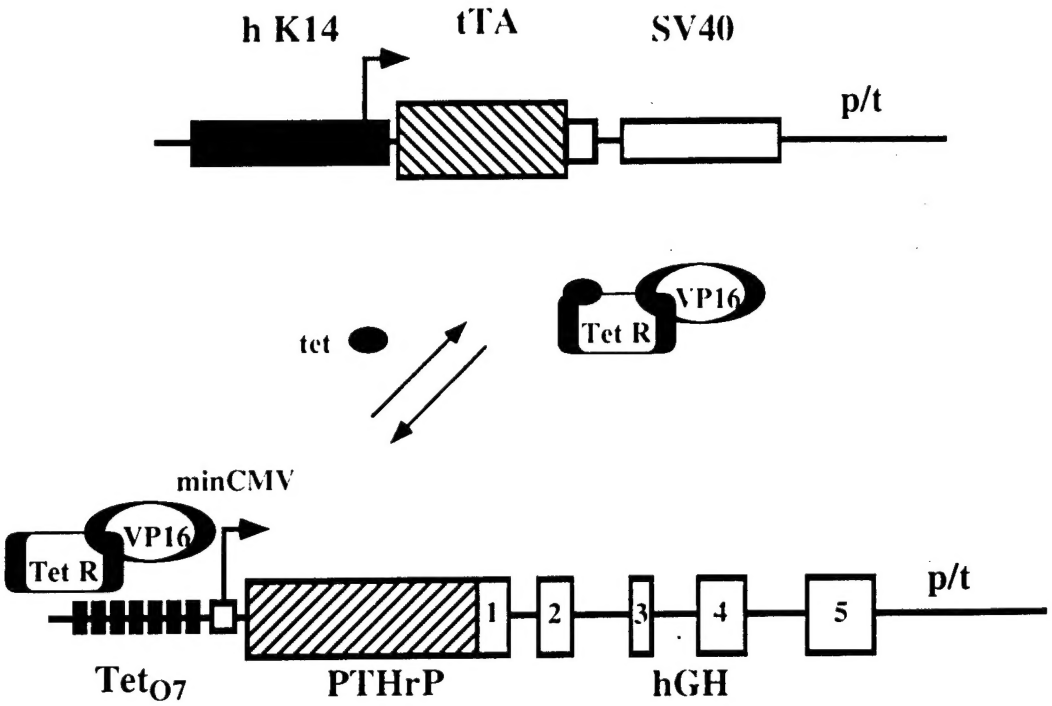


Figure 3. A model for the regulation of mesenchymal cell differentiation by PTHrP on the ventral surface of the embryo. By virtue of PPR1 expression, all of the ventral mesenchymal cells are potentially responsive to PTHrP, but during early mammary development, PTHrP expression is specific to the mammary epithelial bud. PTHrP therefore acts as a dominant signal, contributing to cell fate decisions leading the ventral mesenchyme cells surrounding the mammary epithelial bud to acquire a dense mammary mesenchyme phenotype and the ability to support mammary morphogenesis. In the absence of PTHrP production by the epidermal keratinocytes, the ventral mesenchyme acquires a dermal phenotype.



Tet$_0$ - PTHrP

Figure 4. Tetracycline-regulated PTHrP production using a binary tet-off system. In order to introduce tetracycline regulation into our original K14-PTHrP transgenic model, we created two transgenes. The first, as shown on top, consists of the K14 promoter driving expression of the tet-transactivator protein (tTA), which contains elements of the powerful VP16 transcriptional activator. The second transgene, shown on the bottom, consists of the PTHrP coding region placed downstream of a minimal CMV promoter containing 7 tetracycline operon sequences. The minimal CMV promoter has negligible activity itself, but in the presence of tTA, which binds to the tet-operon sequences, the VP16 domain activates transcription of the PTHrP sequences. However, in the presence of tetracycline, tTA changes conformation and can no longer bind to the tet-operon sequences. Therefore, in this system tetracycline acts as an on-off switch. In animals carrying both transgenes, PTHrP is made in cells which normally express K14 in the absence of tetracycline. When tetracycline is added to the water supply of these mice it turns off expression of the transgene and no PTHrP is produced. Hence, this system now adds temporal regulation of PTHrP production to the pre-existing spatial control of PTHrP production afforded by the K14 promoter.

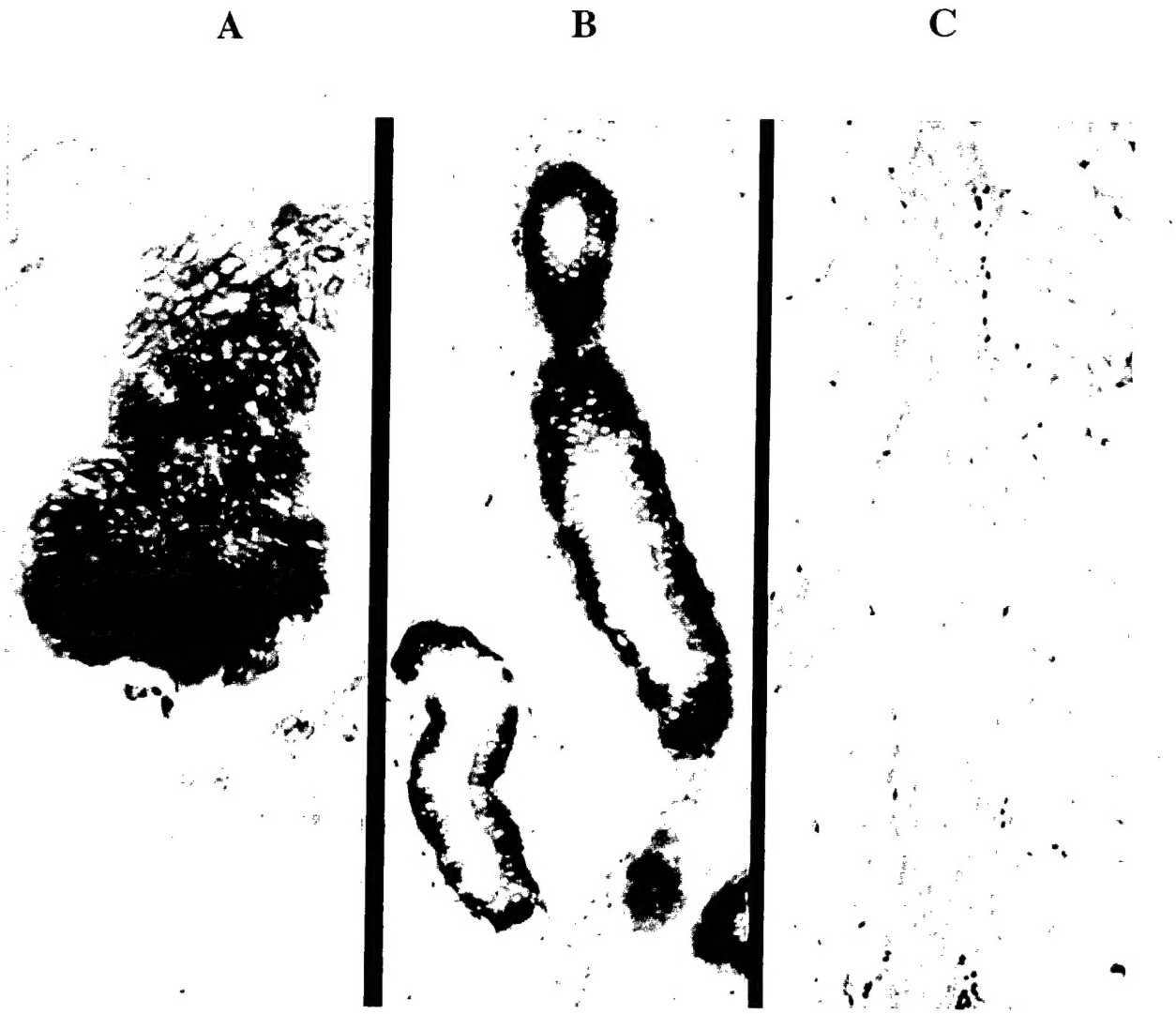


Figure 5. Expression patterns of the K14-tTA transgene in K14-tTA/pTet-β–galactosidase (β-gal) double transgenic mice. Mammary tissue from double transgenic mice was harvested and stained with X-gal. Cells that express the K14-tTA transgene would be expected to turn blue with this procedure, by virtue of them containing β-gal. In the presence of tetracycline, the expression of β-gal, and hence the blue color, should be blocked. (A) The mammary rudiment of a double-transgenic embryo at E15, stained with X-gal. (B) Mammary ducts, stained with X-gal, from an adult double-transgenic mouse. (C) Mammary ducts, stained with X-gal, from an adult double transgenic mouse receiving tetracycline. As one can see, the K14-tTA transgene is expressed within all the mammary epithelial cells before birth (A), and in myoepithelial cells after birth (B). These findings are identical to the expression patterns of the native K14 gene. The addition of tetracycline to the water supply of the mice completely and uniformly suppresses K14-tTA transgene expression in mammary epithelial cells (C).

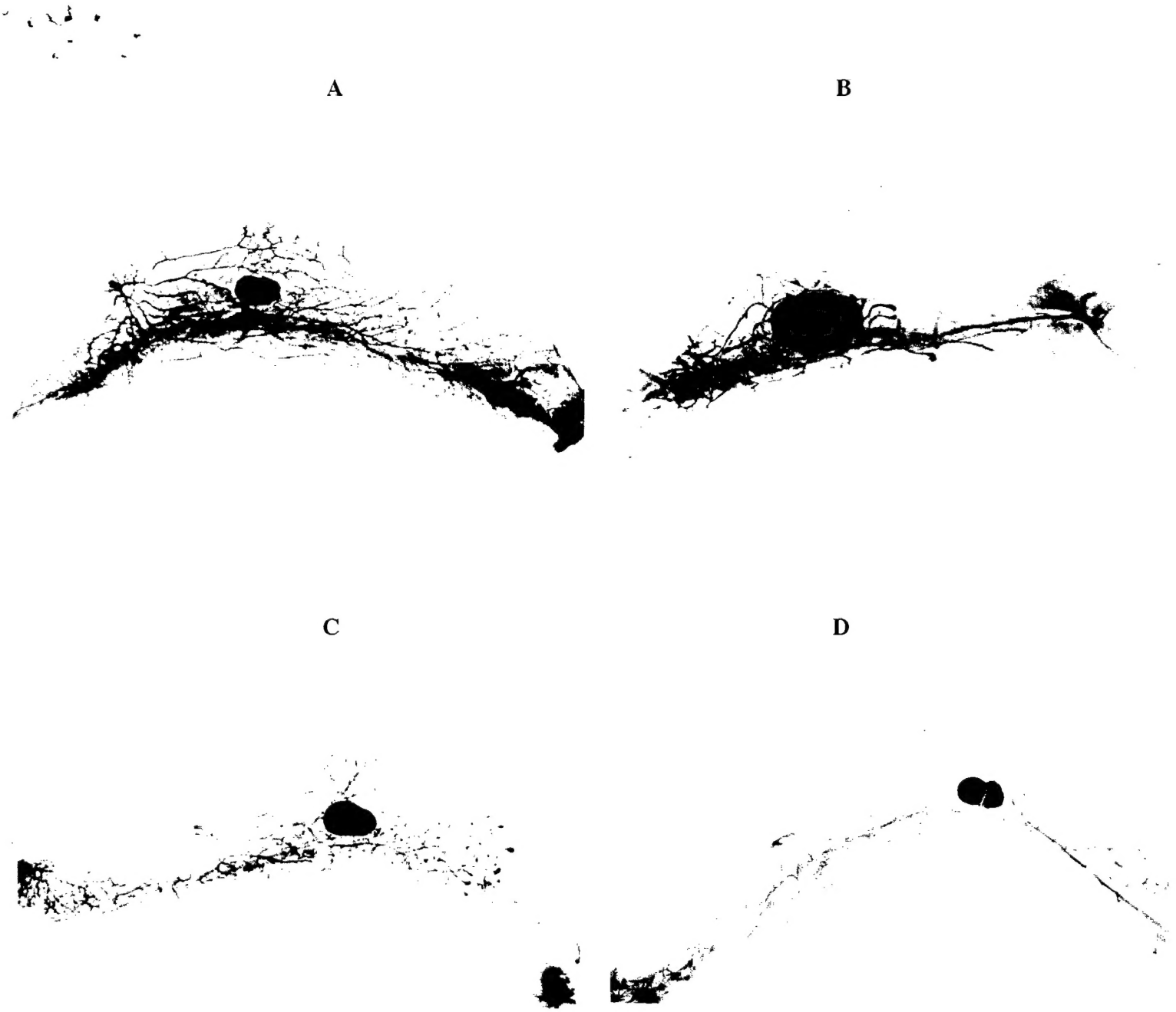


Figure 6. Whole-mount analysis of mammary glands taken from K14-tTA/pTet-PTHrP double transgenic mice exposed to PTHrP overexpression at different points in the mammary gland life-cycle. Whole mammary glands were removed from the mice, fixed in acid-ethanol, stained with carmine-aluminum and defatted before being mounted and photographed. (A) Mammary gland from a double transgenic mouse, never exposed to PTHrP overexpression. This gland appears completely normal. (B) Double transgenic gland always exposed to PTHrP overexpression. This gland recapitulates the original K14-PTHrP transgenic phenotype. There is a delay in the growth of the duct system into

the fat pad, as well as a reduction in the branching complexity. (C) Double transgenic gland exposed to PTHrP overexpression only after birth. This gland has a relatively normal branching pattern, but its growth into the fat pad is delayed. (D) Double transgenic gland exposed to PTHrP overexpression only before birth. This gland has grown into the fat pad at a normal rate. However, there is a severe reduction in the branching complexity of the epithelial ducts. Therefore, it appears that the effects on branching are related to embryonic overexpression of PTHrP, but the effects on ductal elongation are a function of post-natal PTHrP expression.